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Xath5 Participates in a Network of bHLH Genes in the Developing Xenopus Retina

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Summary

We examined the function of basic-helix–loop–helix (bHLH) transcription factors during retinal neurogenesis. We identified *Xath5*, a Xenopus bHLH gene related to Drosophila *atonal*, which is expressed in the developing Xenopus retina. Targeted expression of *Xath5* in retinal progenitor cells biased the differentiation of these cells toward a ganglion cell fate, suggesting that *Xath5* can regulate the differentiation of retinal neurons. We examined the relationship between the three bHLH genes *Xash3*, *NeuroD*, and *Xath5* during retinal neurogenesis and found that *Xash3* is expressed in early retinoblasts, followed by coexpression of *Xath5* and *NeuroD* in differentiating cells. We provide evidence that the expression of *Xash3*, *NeuroD*, and *Xath5* is coupled and propose that these bHLH genes regulate successive stages of neuronal differentiation in the developing retina.

Introduction

Cell type determination and differentiation in several tissues depends upon the function of transcription factors belonging to the basic-helix–loop–helix (bHLH) family. Within a given tissue, multiple bHLH genes can function both coordinately and sequentially to regulate successive steps in the differentiation process. For example, during muscle development, the bHLH factors MyoD and Myf-5 function as determination factors and regulate the transition from mesodermal progenitor cell to determined myoblast (reviewed in Weintraub, 1993; Olson and Klein, 1994). These proteins then activate the expression of myogenin and MRF-4, which act as differentiation factors and promote the formation of postmitotic differentiated myotubes (Weintraub, 1993; Olson and Klein, 1994). Similarly, in Drosophila melanogaster, the proneural genes of the *achaete–scute* complex and *atonal* encode bHLH transcription factors that are required for neural cell fate determination (Campuzano and Modolell, 1992; Ghysen et al., 1993; Jan and Jan, 1993; Jarman et al., 1993; Campos-Ortega, 1994). During peripheral sense organ development, the *achaete* and *scute* genes function together to specify the formation of neural precursors and activate the expression of a downstream bHLH gene, *asense* (Campuzano and Modolell, 1992; Ghysen et al., 1993; Jan and Jan, 1993; Campos-Ortega, 1994).

Numerous homologs of the Drosophila proneural genes have been identified in vertebrates. A subset of these, namely *Mash-1*, *Xash1*, and *Xash3*, are related to the Drosophila *achaete–scute* complex genes (Johnson et al., 1990; Ferreiro et al., 1992; Zimmerman et al., 1993; Turner and Weintraub, 1994), while another subset, including *Math-1*, *Math-2/Nex-1*, *Xath-3*, *Math-4a&b*, *NeuroD*, *NeuroD2*, *neurogenin*, and *X-Ngnr-1a*, are more similar to the Drosophila *atonal* gene (Jarman et al., 1993; Bartholoma and Nave, 1994; Akazawa et al., 1995; Lee et al., 1995; Shimizu et al., 1995; Gradwohl et al., 1996; Ma et al., 1996; McCormick et al., 1996; Takebayashi et al., 1997). Many of these bHLH genes have overlapping expression patterns in the developing vertebrate nervous system. Therefore, vertebrate neurogenesis may also require multiple bHLH factors to regulate successive steps of neuronal development. During primary neurogenesis in developing Xenopus laevis embryos, the bHLH genes *X-Ngnr-1a* and *NeuroD* are sequentially expressed and have been proposed to regulate distinct steps of neuronal development in the neural plate (Ma et al., 1996). During mouse olfactory development, the bHLH genes *Mash1*, *Math4C/neurogenin1*, and *NeuroD* have been shown to be expressed during sequential stages of olfactory neuron differentiation, arguing that in this lineage as well, bHLH genes might function together to regulate successive steps in the neuronal differentiation pathway (Cau et al., 1997). Outside of these examples, the functional relationship between the various bHLH genes in the developing nervous system are not well understood and are likely to vary from tissue to tissue.

The neural retina offers a unique system in which to examine the contribution of multiple bHLH proteins to successive stages of neurogenesis within the same lineage, since all neural retinal cells have been shown to derive from a common pool of multipotential progenitor cells (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Wetts et al., 1989; Turner et al., 1990; Fekete et al., 1994). The process of retinal neurogenesis is particularly accessible to analysis in Xenopus, since the retina continues to grow throughout the life of the animal by the addition of cells at the peripheral edge, a region known as the ciliary marginal zone (CMZ) (Straznicky and Gaze, 1971). The CMZ is organized so that the progressive committment of retinal stem cells to a differentiated neuronal cell fate can be monitored (Dorsky et al., 1995). The most peripheral part of the CMZ consists of self-renewing stem cells, then in the more central CMZ committed retinoblasts, and finally differentiating neurons are represented. In mature retina, the cells in the central retina are postmitotic and fully differentiated, while neurogenesis continues at the margins.

Figure 1. Protein Sequence of Xath5a and Xath5b and Alignment with Other Atonal-Related bHLH Proteins

(A) Alignment of the predicted amino acid sequence of Xath5a and Xath5b. Amino acid identities are indicated by a line. Alignment was performed using the GCG bestfit program.

(B) Alignment of the Xath5a bHLH region with other bHLH proteins. The positions of the basic domain, helix 1, the loop, and helix 2 are shown above the aligned sequences, and the percentage of amino acids that are identical to Xath5a is shown on the right. The atonal-related proteins are grouped together above the consensus, while the achaete–scute-related proteins are shown below. Amino acids that are identical to those in Xath5a are indicated with an asterisk. Amino acids that are conserved in all atonal-related proteins are shown in the consensus. The alignment was performed using the GCG pileup program.

GenBank database accession numbers: Xath5a, U93170; Xath5b, U93171; Atonal, L36646; Math1, D43694; Math2, D44480; Xath3, D85188; Math4A, Y07621; Math4B, Y09167; mNeuroD1, U28068; mNeuroD2, U58471; X-Ngnr-1a, U67778; mNgn, U67776.

The process of retinal neurogenesis has been well studied in the compound eye of Drosophila, where the bHLH gene *atonal* is required for the formation of photoreceptors (Jarman et al., 1994). In the Xenopus retina, several known bHLH genes are expressed, but their role in retinal neurogenesis has not been well characterized. One of these, *Xash3*, is expressed in a subset of neuroblasts within the developing nervous system, while another, *NeuroD*, is generally expressed later, in differentiating neuroblasts and neurons (Zimmerman et al., 1993; Turner and Weintraub, 1994; Lee et al., 1995). It has been proposed that *Xash3* regulates an early step and *NeuroD* a later step during neurogenesis, although this has never been demonstrated for cells within the same lineage (Ferreiro et al., 1994; Turner and Weintraub, 1994; Anderson, 1995; Lee et al., 1995). In contrast to the neural plate, in which *Xash3* and *NeuroD* are expressed in nonoverlapping patterns and hence may function to regulate the development of distinct subsets of neurons (Zimmerman et al., 1993; Turner and Weintraub, 1994; Anderson, 1995; Lee et al., 1995), the expression of both *Xash3* and *NeuroD* in the developing Xenopus retina suggests that these two proteins could function to regulate different stages of neurogenesis within the same lineage.

We describe here *Xath5*, an *atonal*-related gene from Xenopus, which has a specific function in regulating retinal neurogenesis. *Xath5* is expressed in the developing neural retina and was able to promote the differentiation of retinal ganglion cells when expression was targeted to retinal precursor cells by lipofection or 16-cell RNA injection. We compared the expression of *Xath5* to that of *Xash3* and *NeuroD* during retinal neurogenesis and found that *Xash3* was expressed early in retinoblasts prior to the onset of neuronal differentiation, and *NeuroD* and *Xath5* were expressed later, in differentiating neuroblasts. During early retinal development, as well as in the CMZ of the retina, *Xath5* and *NeuroD* expression commenced in the same cells, suggesting that they function at a similar stage of retinal cell differentiation. When the functional relationship between *Xash3*, *NeuroD*, and *Xath5* was examined in neural plate stage embryos, we found that *Xash3* overexpression could promote ectopic expression of *NeuroD* but then limit activation of genes downstream of *NeuroD*. This suggests that *Xash3* may regulate the responsiveness of cells to *NeuroD* expression, thus regulating the timing of progenitor cell differentiation. *NeuroD* overexpression could activate *Xath5* expression in a region restricted to the anterior neural ridge, a region fate mapped to produce retina and olfactory placode (Eagleson and Harris, 1989; Eagleson et al., 1995) where *Xath5* is later expressed. In turn, *Xath5* could activate *NeuroD* expression throughout the neural plate and ectoderm. Based upon these observations, we propose that the expression of *Xash3*, *NeuroD*, and *Xath5* is coupled and that these bHLH genes regulate successive stages of neuronal differentiation in the developing Xenopus retina.

Results

Molecular Characterization of *Xath5*, a Vertebrate *atonal* Homolog

To identify candidate genes involved in regulating retinal neurogenesis, we isolated a homolog of the Drosophila *atonal* gene from Xenopus. Degenerate PCR primers directed against the conserved bHLH domain were designed based upon amino acid identities between Drosophila atonal and Math1 (Jarman et al., 1993; Akazawa et al., 1995). PCR was performed from a stage 28–30 Xenopus head cDNA library (gift from R. Harland), and the PCR fragments obtained were subcloned and sequenced. A PCR fragment showing strong homology to Drosophila *atonal* throughout the bHLH region was used

as a probe to screen the same cDNA library. Two independent cDNA clones 2.14 kb and 2.29 kb in length were obtained that were 70% identical at the nucleotide level. Since these two genes likely arose due to the ancestral duplication of the Xenopus genome, they were named *Xath5a* and *Xath5b*, in accord with the convention for *atonal*-related genes. Both cDNAs contained a 417 nucleotide open reading frame encoding predicted proteins of 138 amino acids and 15 kDa. The codon for the putative start methionine in each gene was preceded by a stop codon in all three frames. The proteins encoded by *Xath5a* and *Xath5b* were 90% identical to each other (Figure 1A). When in vitro translation reactions were performed using the full-length cDNAs or precise subclones of the protein coding regions, proteins of approximately 15 kDa were obtained, although it was observed that in vitro translated Xath5b protein had consistently reduced electrophoretic mobility in SDS-PAGE gels relative to Xath5a protein (data not shown). *Xath5a* and *Xath5b* exhibited identical expression patterns at all stages examined (described below) and caused similar phenotypic effects when overexpressed (see below). The genes will therefore be described simply as *Xath5*.

The Xath5 protein shares 70% amino acid identity with the Drosophila atonal protein within the bHLH domain and 100% identity within the basic domain (Jarman et al., 1993). Alignment of the bHLH amino acid sequence of Xath5a with that of other bHLH proteins showed that Xath5 belongs to an atonal-related family of proteins, with Xath5 being among the most highly related to Drosophila atonal. All proteins within this family have bHLH domains with striking conservation in amino acid composition (Figure 1B). Twenty-six out of 56 residues within the bHLH domain are identical in all atonal family members (shown in the consensus, Figure 1B). Five of these conserved amino acids, concentrated in the basic domain and helix 1, are characteristic of the atonal protein family when compared with achaete–scute-related proteins (underlined in Figure 1B). In addition, the atonal-related proteins contain a three amino acid insert in the basic domain (LAA in Xath5) that is not found in the achaete–scute-related proteins. In Drosophila, it has been shown that amino acid differences in the basic domain between atonal and the achaete–scute proteins are responsible for determining neuronal cell type (Chien et al., 1996). In vertebrates, the functional significance of these residues has yet to be determined. Besides the bHLH domain, Xath5 shows no significant homology to any atonal-related proteins or any other proteins in the database.

The Expression of *Xath5* during Xenopus Development

The expression of *Xath5* mRNA during Xenopus embryonic development was examined by whole-mount in situ hybridization. *Xath5* was first detected at the late neural fold stage (stage 17) as two symmetric patches of expression in the anterior neural ectoderm in the region of the presumptive olfactory placodes (Figure 2A, stage 21 shown) (Eagleson and Harris, 1989; Eagleson et al., 1995). This is prior to the formation of the olfactory placodes themselves, which occurs at around stage 23 (Klein and Graziadei, 1983). *Xath5* expression began in the pineal at stage 23, then shortly after, at stage 24, punctate expression was also detected in the eye, coincident with the reported onset of neuronal differentiation in the retina (data not shown; Holt et al., 1988). At stage 24, there was often a slight dorsal-to-ventral gradient of *Xath5* expression in the eye (data not shown), which is consistent with neurogenesis commencing slightly earlier in the dorsal retina than in the ventral retina at this stage (Holt et al., 1988). By stage 27 (tailbud stage), strong expression was observed in all three regions: the retina, the olfactory placodes, and the pineal (Figure 2B). The olfactory and pineal expression had diminished by stage 32, but strong expression continued in the retina at least through stage 46 (Figure 2D).

Expression of *Xath5* in the Developing Neural Retina

To understand what role *Xath5* might play in regulating retinal neurogenesis, the expression of *Xath5* was examined more carefully in the developing eye. At stage 32, when neurons throughout the retina are differentiating, *Xath5* expression was distributed throughout the neural retina (Figure 2C) and could be seen in columnar neuroepithelial-like cells (Figure 2E). At later stages, *Xath5* expression was no longer observed in the central retina and became restricted to the margins (Figure 2D, stage 46 shown). At stage 40, when most cells in the central retina are postmitotic, *Xath5* expression was largely observed in the CMZ, with the exception of a few cells in the central retina that probably represent late-born retinal cells (Figure 2F). By stage 42, when the retina had achieved its mature laminar organization, *Xath5* expression was almost completely restricted to the CMZ (Figure 2G). *Xath5* expression was not observed at the peripheral edge of the CMZ (Figures 2F and 2G), where stem cells are found (Dorsky et al., 1995). In the CMZ, some of the *Xath5*-expressing cells were proliferative and labeled with bromodeoxyuridine (BrDU) (M. P. and W. A. H., submitted). Therefore, in the developing retina, *Xath5* expression marked a population of cells that was in transition from proliferating retinoblast to differentiating retinal neuron.

Overexpression of *Xath5* Promotes Neuronal Differentiation

A number of *atonal*-related proteins, including NeuroD, NeuroD2, mNgn, X-Ngnr-1a, and Xath3, promote ectopic neurogenesis throughout the ventral and lateral ectoderm when overexpressed by converting epidermal cells into neurons (Lee et al., 1995; Ma et al., 1996; McCormick et al., 1996; Takebayashi et al., 1997). This suggests that these bHLH proteins can activate a program of neurogenesis within nonneuronal cells and may play an important role in regulating neurogenesis during normal development. Since Xath5 shares sequence homology with these atonal-related proteins, we tested whether it has similar activity. *Xath5* RNA was injected into one cell of a two-cell stage embryo. The embryos were collected at stage 14 and probed by whole-mount in situ hybridization for the expression of *N-tubulin*, a

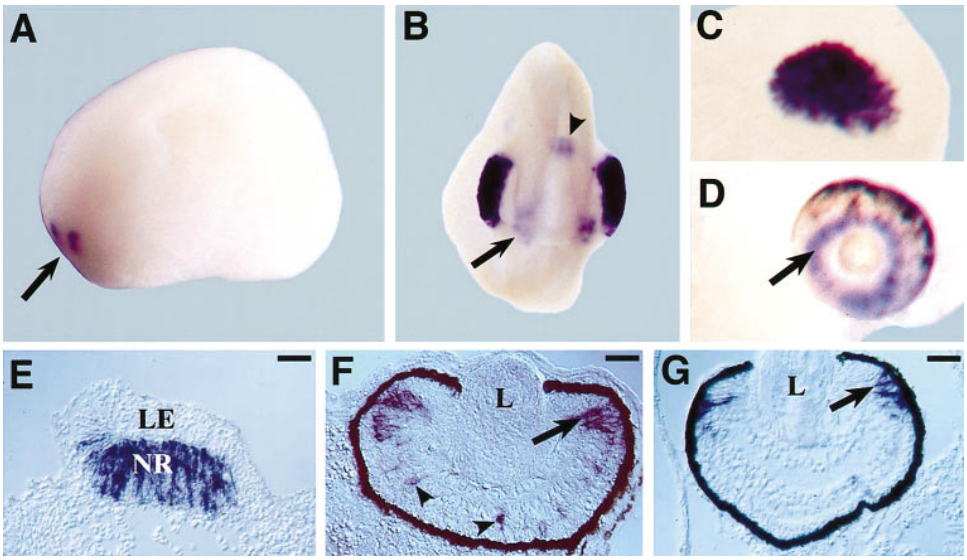

Figure 2. *Xath5* Is Transiently Expressed in the Developing Olfactory Placodes, Retina, and the Pineal Gland

In situ hybridization was performed on whole-mount Xenopus embryos or sections of developing retina using a DIG-labeled *Xath5* probe.

(A) An anterolateral view of a stage 21 embryo showing *Xath5* expression in two patches at the anterior end of the embryo (arrow), in the region of the presumptive olfactory placodes.

(B) Anterior view of a stage 27 embryo showing expression in the eye, pineal gland (arrowhead), and olfactory placodes (arrow).

(C) In a lateral view of a stage 32 embryo, *Xath5* expression could be seen throughout the retina.

(D) At stage 46, *Xath5* expression was completely restricted to the margins of the retina (arrow). The whole retina is demarcated by pigment epithelium (brown).

(E) In a section from a stage 32 embryo, *Xath5* expression was seen in columnar neuroepithelial-like cells throughout the presumptive neural retina.

(F) Later, at stage 40, *Xath5* expression was restricted to the CMZ (arrow) and to a few cells in the central retina (arrowheads), probably late-born cells.

(G) Finally, at stage 42, *Xath5* was solely restricted to cells in the CMZ (arrow). The embryos shown in (B) and (C) were cleared using a 2:1 mixture of benzyl benzoate/benzyl alcohol. LE, presumptive lens epithelium; NR, presumptive neural retina; L, lens. Scale bar = 50 μm.

neuronal-specific marker that marks developing neuroepithelial cells (Richter et al., 1988). Coinjection of RNA for β-galactosidase permitted identification of the injected side by X-Gal staining. Overexpression of *Xath5* promoted the formation of ectopic *N-tubulin*-positive cells throughout the neural plate (Figure 3Aii, 50/55 embryos examined), similar to that described for *NeuroD* (Lee et al., 1995; Figure 6Ci). Ectopic *N-tubulin*-positive cells were never observed in *lacZ*-injected embryos (Figure 3Ai). There was no difference in activity between the two forms of *Xath5*. We also observed the production of ectopic N–CAM-positive cells, with neuronal, process-bearing morphology throughout the ventral and lateral ectoderm in stage 21 embryos by immunohistochemistry using an antibody specific for N–CAM (data not shown; 206/230 embryos examined). These results support a role for *Xath5* in regulating neurogenesis in the developing embryo.

Targeted Expression of *Xath5* in Retinal Progenitor Cells Promotes Retinal Ganglion Cell Fate

During retinal neurogenesis, the different cell types of the retina are born in a rough, overlapping sequence that is conserved across species. Retinal ganglion cells are born first, followed by horizontal cells, cone photoreceptors, amacrine cells, Mueller glial cells, bipolar cells, then rod photoreceptors (reviewed in Reh, 1992). *Xath5* is normally expressed in the developing retina at the time of retinal progenitor cell differentiation and may therefore play an important role in regulating the differentiation of these cells. To test this idea, we targeted *Xath5* to the developing retina by in vivo lipofection of *Xath5* DNA into the optic vesicles of stage 18 embryos. DNA for green fluorescent protein (GFP) was cotransfected, permitting identification of transfected cells in stage 41 retina, by which time most cells in the central retina are postmitotic and fully differentiated (Holt et al., 1988). Holt et al. (1990) have shown that there is a very high rate of cotransfection (85%–100%) when two genes are transfected simultaneously. In some sections, we confirmed that *Xath5* was being expressed by staining for the *myc* epitope tag (data not shown).

Retinal cells transfected with *Xath5* showed a significant bias toward a retinal ganglion cell fate (Figure 3B). On average, *Xath5*-transfected embryos showed a 2.7-fold increase in the representation of ganglion cells as compared to embryos transfected with GFP alone. The GFP-labeled cells in the ganglion cell layer had a morphology characteristic of ganglion cells, and in many cases, the axon leaving the retina and projecting toward the optic nerve was labeled. In addition, a subset of embryo sections was stained with an antibody for islet-1, a ganglion cell marker (Dorsky et al., 1997), confirming that the *Xath-5*-transfected cells in the ganglion cell layer were indeed ganglion cells (data not shown). This dramatic increase in retinal ganglion cells, the first retinal

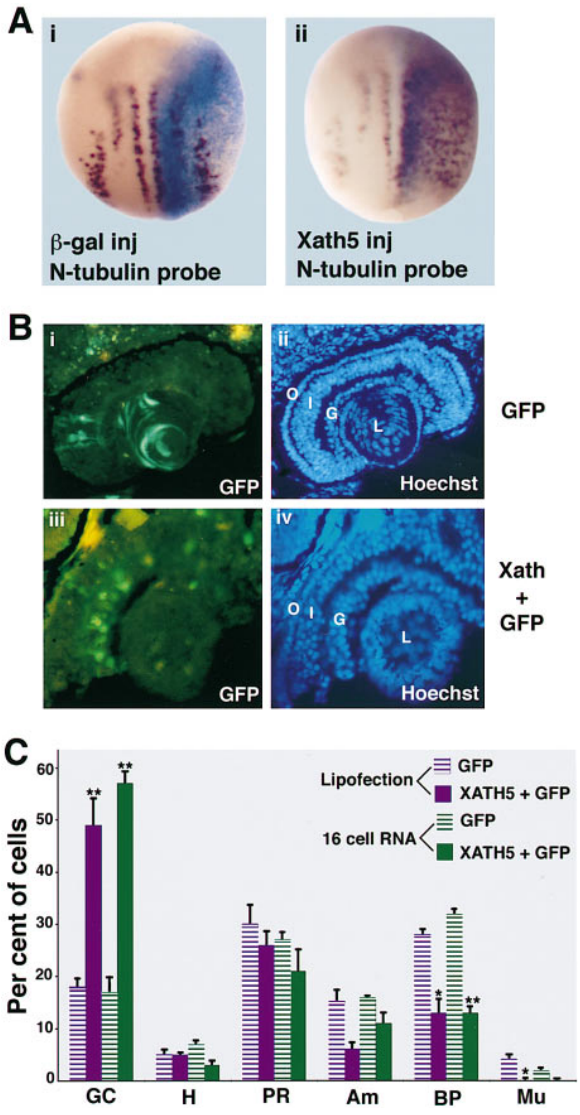

Figure 3. *Xath5* Overexpression Promotes Neurogenesis and Retinal Ganglion Cell Differentiation

(A) RNA was injected into one cell of a two-cell stage Xenopus embryo. Stage 13 embryos (neural plate stage) were then stained with X-Gal to detect β-galactosidase expression (aqua), followed by whole-mount in situ hybridization for *N-tubulin* (dark purple). The embryos are shown in a dorsal view, with anterior at the top and the injected side on the right. (i) A control embryo injected with RNA for β-galactosidase showing normal *N-tubulin* expression in the developing nervous system. (ii) An embryo injected with RNA for *Xath5* and β-galactosidase showing ectopic expression of *N-tubulin* on the injected side, indicating the formation of ectopic neuroepithelial cells.

(B) Retinal lipofection: embryos were transfected at stage 18 in the region of the optic vesicle with a combination of lipofectin plus GFP DNA alone (i and ii) or plus *Xath5* and GFP DNA together (iii and iv). At stage 41, the embryos were cryostat sectioned and the nuclei stained with Hoechst to identify retinal cell layers. The layers are indicated as follows: G, ganglion cell layer; I, inner nuclear layer; O, outer nuclear layer; L, lens. (i) and (ii) show a retinal section from an embryo transfected with GFP DNA alone, showing GFP-labeled cells (i) and the corresponding retinal cell layers, stained with Hoechst (ii). In this section, the labeled cells are found in the inner nuclear layer (I) and outer nuclear layer (O), with some lens cells labeled as well (L). (iii) and (iv) show a retinal section from an embryo transfected with *Xath5* DNA and GFP DNA, showing cells labeled with GFP (iii) and corresponding retinal cell layers stained with Hoechst (iv). In the *Xath5* plus GFP-transfected retina, the majority of GFP-positive cells are found in the retinal ganglion cell layer (G).

(C) Percentage of retinal cell types labeled by following misexpression of GFP (hatched bars) or *Xath5* plus GFP (solid bars). Two different methods of misexpression were used: DNA lipofection at stage 18 (purple) or 16-cell RNA injection into blastomere D.1.1 (green). Transfection of *Xath5* plus GFP (solid purple bars) produced significantly more ganglion cells and significantly fewer amacrine, bipolar, and Mueller cells than transfection of GFP alone (purple hatched bars). n = 472 cells from five embryos (GFP) and 939 cells from six embryos (*Xath5* plus GFP). Similarly, injection of *Xath5* and GFP RNA at the 16-cell stage (solid green bars) resulted in significantly more ganglion cells and significantly fewer bipolar cells than injection of GFP RNA alone (hatched green bars). n = 539 cells from five embryos (GFP) and 466 cells from five embryos (*Xath5* plus GFP).

The percent representation of each cell type was calculated as a weighted average. Error represents SEM; single asterisk, $p < 0.02$, double asterisks, $p < 0.001$ by Student's t test. GC, ganglion cells; H, horizontal cells; PR, photoreceptor cells; Am, amacrine cells; BP, bipolar cells; Mu, Mueller cells. Cell types are represented from left to right in rough order of birth.

cell type to be generated, was at the expense of later-born cell types, namely amacrine, bipolar, and Mueller cells, which showed significant decreases in representation compared with controls (Figure 3B). This observation is consistent with a model whereby transfection of *Xath5* promotes early differentiation of retinal progenitor cells at a time when the cues within the retina are specifying early-born cell types, such as retinal ganglion cells. Not all cells may respond uniformly to *Xath5* expression, since there is considerable temporal overlap in the generation of the different cell types within the Xenopus retina, so that more than one cell type is being generated at any given time (Holt et al., 1988). In addition, as cells begin to differentiate, they may generate signals that either limit the ability of surrounding cells to take on similar fates or instruct them to take on different fates.

To confirm that *Xath5* was able to bias the differentiation of retinal cells toward a ganglion cell fate, we injected a combination of GFP RNA and *Xath5* RNA at the 16-cell stage into blastomere D.1.1, which contributes to roughly 50% of the cells of the ipsilateral retina and 7% of the contralateral retina (Moody, 1987; Huang and Moody, 1993). Clones of misexpressing cells were located in sections of stage 41 retina and the cell types within the clone identified. Eye development was often reduced on the injected side of the embryo, as has been reported for *NeuroD* overexpression (Lee et al., 1995; Hirsch and Harris, 1997), in which case labeled cells in the contralateral retina were scored. The profile of retinal cell types labeled using this method was very similar to that observed using the lipofection method (compare Figures 3A and 3B). On average, cells derived from the *Xath5*-injected blastomeres showed a 3.4-fold increase in the representation of retinal ganglion cells, as compared with the cells derived from blastomeres where GFP was injected alone (Figure 3C). Once again, the increase in representation of retinal ganglion cells was at the expense of the later-born cell types (Figure 3C).

To determine whether the ability to promote retinal ganglion cell differentiation is shared by bHLH proteins other than *Xath5*, we targeted expression of *NeuroD* to

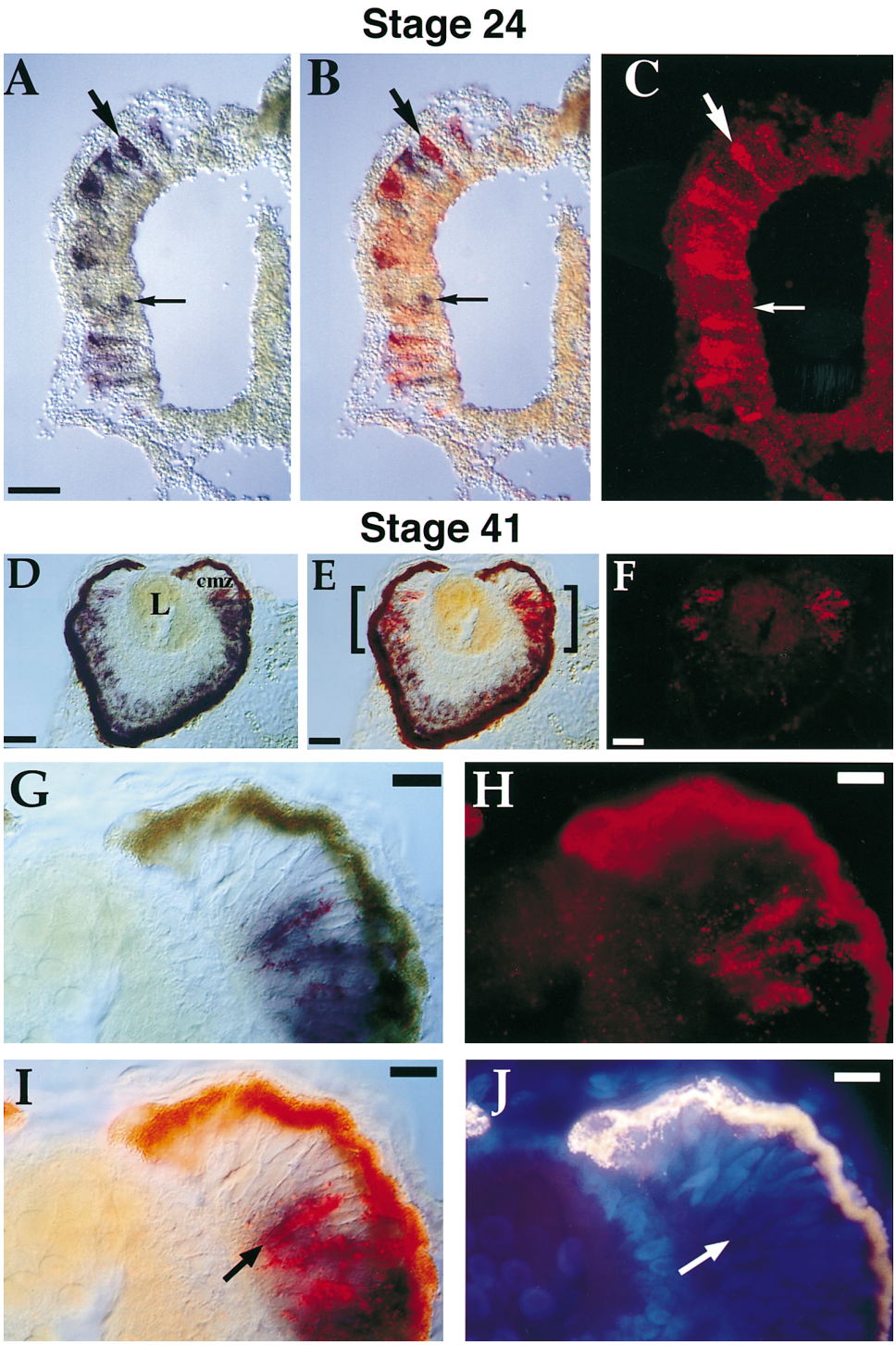

Figure 4. *Xath5* and *NeuroD* Commence Expression at the Same Stage of Retinal Neurogenesis

Double in situ hybridizations were performed on sections of Xenopus retina using DIG-labeled *NeuroD* probe and fluorescein-labeled *Xath5* probe. *NeuroD* expression was viewed under visible light (deep purple), and *Xath5* expression was viewed by fluorescence (red). The dorsal side is on the right in all sections.

(A)–(C) show the same section from a stage 24 Xenopus embryo. (A) *NeuroD* expression was seen in scattered cells throughout the early neural retina. (C) *Xath5* expression was observed in a similar population of retinal cells. (B) Double labeling of the retina demonstrates that most early retinal cells express both *NeuroD* and *Xath5* (a double-labeled cell is indicated by the large arrow). Occasionally, cells expressed *NeuroD* but not *Xath5* (an example is indicated by the small arrow).

(D)–(J) show the same section from a stage 41 Xenopus embryo. (D) *NeuroD* expression was seen in cells in the CMZ and in the central retina. The dense outermost layer seen in these sections is the pigment epithelium, unlabeled for both *NeuroD* and *Xath5*. (F) *Xath5* labeling was restricted to the more central part of the CMZ, and occasional labeled cells were seen in the central retina. (E) Double labeling of retina demonstrated that *Xath5* and *NeuroD* expression overlapped in the the CMZ (indicated by square brackets).

retinal progenitors using in vivo lipofection (203 transfected cells; five embryos). Strikingly, retinal cells transfected with DNA for *NeuroD* showed no significant change in the representation of ganglion cells or horizontal cells relative to controls. However, there was a 2-fold increase in amacrine cells and a 2.7-fold increase in bipolar cells when compared to controls. These increases were statistically significant ($p < 0.02$, Student's t test) and paralleled a significant decrease in the representation of photoreceptors, which were reduced in number to one-fourth of controls, and a complete loss in the representation of Mueller cells. All cells that were counted expressed the NeuroD protein, as determined by antibody staining at stage 41 for the *myc* epitope tag. This eliminated the possibility that the absence of an effect on retinal ganglion cell differentiation was due to a lack of protein expression or loss of *NeuroD*-expressing cells.

These results suggest that *Xath5* and *NeuroD* have nonidentical roles during retinal neurogenesis, despite their sequence similarity (Figure 1B), their coexpression during retinal cell differentiation (see below and Figure 4), and their common ability to promote ectopic neurogenesis (Figures 3Aii and 6Ci). Embryos injected with *NeuroD* RNA showed severe disruption of eye formation on the ipsilateral side, likely due to suppression of pax-6 expression (Hirsch and Harris, 1997), and labeled cells were poorly represented in the contralateral retina, thus limiting a comparison between *Xath5* and *NeuroD* using this method. Nevertheless, the fact that *Xath5* had a specific effect on retinal ganglion cell differention using two different methods supports a role for *Xath5* in promoting the differentiation of retinal progenitor cells.

Xath5 and *NeuroD* Expression Commences at the Same Stage of Retinal Neurogenesis

Both *Xath5* and *NeuroD* are expressed in the developing Xenopus retina beginning at about stage 24, a time when the neurons of the retina first become postmitotic and begin differentiating (Lee et al., 1995; data not shown). To determine whether *NeuroD* and *Xath5* function at the same step or at sequential steps in the development of retinal neurons, we compared the expression of these two genes by double in situ hybridization on sections of Xenopus retina (Figure 4). At stage 24, we observed that most cells expressing *NeuroD* (Figure 4A) also expressed *Xath5* (Figures 4B and 4C). Occasionally, cells were found to be *NeuroD*- but not *Xath5*-positive (Figure 4B), suggesting either that *Xath5* expression had not yet been activated or that a subset of cells will only express *NeuroD* and not *Xath5*.

By stage 41, the neurons in the central retina are postmitotic and fully differentiated, while proliferation and differentiation continues in the CMZ, allowing the sequential steps of neurogenesis to be monitored from peripheral to central retina (Straznicky and Gaze, 1971). Double in situ hybridization demonstrated that the expression of *NeuroD* and *Xath5* commenced in the same cells of the CMZ, in columnar neuroepithelial-like cells (Figures 4D–4J). Both *NeuroD* and *Xath5* were excluded from the peripheral edge of the CMZ (Figures 4D–4F), indicating that they function in differentiating neuroblasts rather than in proliferating stem cells. *Xath5* expression ceased in the differentiated cells of the central retina (Figures 2F, 2G, and 4F), while *NeuroD* expression was maintained in cells of the outer part of the inner nuclear layer and in cells of the photoreceptor layer (Figure 4D).

Cross-Activation of *NeuroD* and *Xath5* Expression

Since *Xath5* and *NeuroD* commenced expression at the same stage of retinal cell differentiation in the CMZ, we postulated that their expression may be coupled at the transcriptional level. Since both *NeuroD* and *Xath5* began expression at neurula stages, we examined the functional relationship between these genes by examining their ability to cross-activate each other's expression in neural plate stage embryos. This analysis was not possible in the developing retina, since at later stages, overexpression of either *NeuroD* or *Xath5* suppressed eye formation on the injected side, likely due to suppression of pax-6 expression (Hirsch and Harris, 1997). We therefore overexpressed *Xath5* by RNA injection at the two-cell stage and performed whole-mount in situ hybridization on stage 14 embryos using a *NeuroD* probe. We observed strong induction of *NeuroD* expression throughout the injected side of the embryos (Figure 5B, 34/34 embryos examined), in a pattern that overlapped the expression of β-galactosidase.

Conversely, embryos that had been injected with *NeuroD* mRNA showed activation of *Xath5* expression on the injected side, but this was restricted to the anterior neural ridge, a region of the neural plate fate mapped to give rise to retina and olfactory placodes, sites of future *Xath5* expression (Figure 5D, 54/55 embryos examined) (Eagleson and Harris, 1989; Eagleson et al., 1995). In stage 21 embryos, *Xath5* expression on the injected side continued to be largely restricted to the anterior neural tube (Figure 5E) and appeared to represent an expansion of the normal expression of *Xath5* in the olfactory placodes. Occasionally, with longer staining, some weaker activation of expression was also observed within the ectoderm of the injected side (data not shown). Activation of *Xath5* expression was not specifically detected in the eye, since this structure was severely reduced in size or absent on the injected side of *NeuroD*-injected embryos. Since the ability of *NeuroD*

(G)–(J) show a higher magnification of the dorsal CMZ from the section depicted in (D–F), photographed after staining the section with Hoechst dye. The Hoechst dye binds to the pigment epithelial layer (dark brown outer layer in [G]), leading to strong nonspecific fluorescence in this part of the retina (seen in [H]–[J]). (G) Exposure under visible light shows *NeuroD* expression in deep purple. Even without fluorescence, a red precipitate of the fast red substrate, overlapping exactly the deep purple staining, reveals *Xath5* expression. (H) Exposure under fluorescence reveals *Xath5* expression. (I) Double labeling shows that *NeuroD* and *Xath5* commence expression in the same cells and that the expression of *Xath5* exactly overlaps that of *NeuroD*. Neither *Xath5* nor *NeuroD* were expressed in the most peripheral part of the CMZ. (J) Visualization of nuclei under UV light after Hoechst dye labeling. Arrow in (I) indicates a double-labeled cell; arrow in (J) indicates the corresponding nucleus. cmz: ciliary marginal zone; L: lens.
Scale bar in (A) and (D)–(F) = 50 μm; scale bar in (G)–(J) = 20 μm.

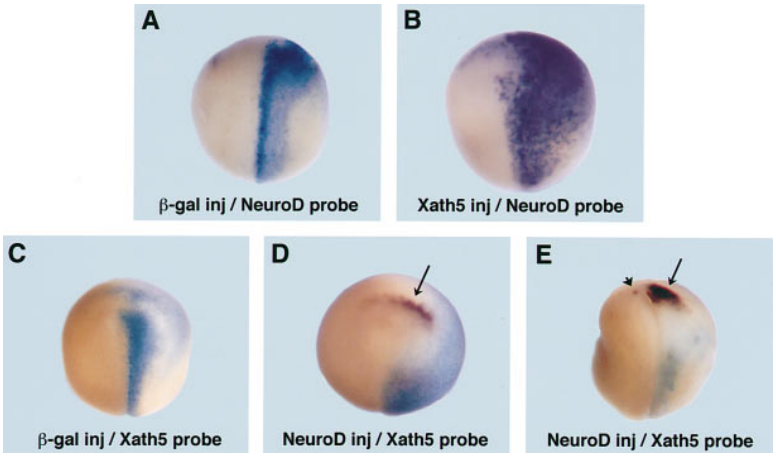

Figure 5. *Xath5* and *NeuroD* Can Activate Each Other's Expression

Embryos were injected at the two-cell stage with RNA for β-galactosidase combined with either *Xath5* or *NeuroD*. The embryos were cultured until stage 13 (A–D) or stage 21 (E), stained with X-Gal (aqua), and then probed by in situ hybridization for expression of *NeuroD* or *Xath5*, respectively (dark purple). The embryos are shown in a dorsal view with anterior at the top. The injected side is always on the right.

(A) A control embryo injected with RNA for β-galactosidase, showing *NeuroD* expression in the developing trigeminal ganglia.

(B) An embryo injected with *Xath5* and β-galactosidase, showing activation of *NeuroD* expression throughout the ectoderm of the injected side (dark purple).

(C) A control embryo injected with RNA for β-galactosidase and probed for *Xath5* expression demonstrated that *Xath5* is not expressed at this stage.

(D) An embryo injected with RNA for *NeuroD* and β-galactosidase showing that activation of *Xath5* expression was restricted to the anterior edge of the neural plate (arrow).

(E) At stage 21, activation of *Xath5* expression in *NeuroD*-injected embryos was concentrated at the anterior part of the neural tube (small arrow) in a domain that corresponds to the normal region of expression at this stage, as seen on the uninjected side (arrowhead).

to activate *Xath5* expression was restricted to the anterior neural ridge and anterior neural tube, either availability of positive cofactors or absence of inhibitory factors must restrict the ability of *NeuroD* to promote *Xath5* expression to those lineages in which *Xath5* is normally expressed. Since *NeuroD* generally has a broader domain of expression during normal development, there may be less restriction in the ability of *Xath5* to promote ectopic activation of its expression.

In summary, these results indicate that during development, the coexpression of *Xath5* and *NeuroD* during retinal neurogenesis could be ensured by cross-activation of the other's expression. It remains to be determined whether such cross-activation occurs during normal retinal development. In fully differentiated neurons of the central retina, additional factors must either selectively limit the expression of *Xath5* or maintain the expression of *NeuroD*.

Xash3 Is Expressed before *NeuroD* and *Xath5* during Retinal Neurogenesis

What are the signals that initiate the expression of *Xath5* and *NeuroD* during retinal development? *Xash3* is a Xenopus *achaete–scute* homolog that is also expressed in the developing retina (Turner and Weintraub, 1994). During nervous system development, *Xash3* expression is largely restricted to proliferating populations of precursor cells (Turner and Weintraub, 1994). To determine whether *Xash3* functions at an earlier step in the differentiation of retinal cells, we compared retinal expression of *Xash3* to that of *NeuroD* and *Xath5* by whole-mount in situ hybridization of stage 21 Xenopus embryos prior to the onset of neuronal differentiation in the retina (Holt et al., 1988). *Xash3* was strongly expressed in the prospective retina at this stage, consistent with expression in proliferating precursor cells (Figure 6Ai). Neither *Xath5* nor *NeuroD* was detected in the developing eye at this stage, demonstrating that they function at a later stage of retinal development (Figures 6Aii and 6Aiii). In stage 41 Xenopus retina, *Xash3* was expressed in early retinoblasts at the margins of the retina and showed some overlap in expression with *NeuroD* and *Xath5*. *Xash3* was not observed in differentiated cells of the central retina (M. P. and W. A. H., submitted).

Xash3 Expression Can Activate Expression of *NeuroD* but Suppresses Its Downstream Effects

Since *Xash3* is implicated in regulating an early step during retinal neurogenesis and *NeuroD* and *Xath5* a later step, there must be coupling between these successive stages of neuronal development. This could be achieved if *Xash3* were to activate the expression of either *NeuroD* or *Xath5*, or both. To test this hypothesis, we injected *Xash3* RNA into cleavage-stage embryos then assayed for the expression of *NeuroD* or *Xath5* by whole-mount in situ hybridization in stage 14 embryos. We found that overexpression of *Xash3* caused ectopic expression of *NeuroD* (Figure 6Bi, 68/73 embryos examined). *NeuroD* expression was activated throughout the injected side of the embryo and generally overlapped completely the expression of β-galactosidase (Figure 6Bi).

However, *Xash3* overexpression did not cause ectopic expression of *Xath5* (Figure 6Bii, 0/57 embryos examined), arguing that *Xash3* was not able to activate expression of this gene directly. Since we have observed that *Xash3* overexpression can activate expression of *NeuroD*, and *NeuroD* overexpression can activate expression of *Xath5*, one might expect secondary activation of *Xath5* expression following *Xash3* overexpression. Since this was not observed, it is possible that inhibitory factors activated by *Xash3* overexpression may limit the ability of *NeuroD* to activate target genes such as *Xath5*. To test this idea, we coinjected RNA for *Xash3* and *NeuroD* into one cell of a two-cell embryo then assayed for either ectopic *N-tubulin* expression or activation of *Xath5*. At doses of *Xash3* RNA that have previously been shown to suppress the formation of *N-tubulin*-positive neuroepithelial cells (see Figure 6Ciii) (Chitnis and Kintner, 1996), we found that *Xash3* was able to inhibit the ability of *NeuroD* to promote both ectopic *Xath5* expression (Figure 6Cv) as well as ectopic neurogenesis (Figure

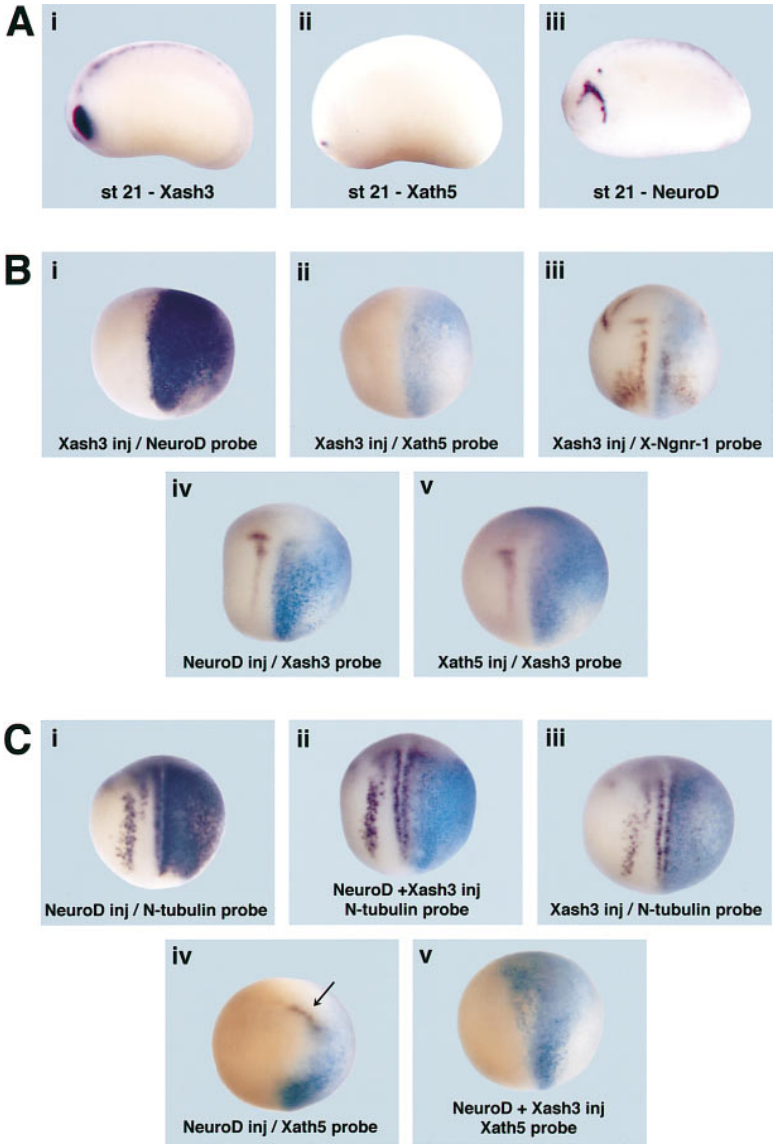

Figure 6. *Xash3* Expression in the Developing Retina Precedes that of *NeuroD* and *Xath5*, and Overexpression of *Xash3* Activates Expression of *NeuroD* but Suppresses Its Downstream Effects

(A) Lateral view of uninjected stage 21 embryos probed by whole-mount in situ hybridization for expression of (i) *Xash3*, (ii) *Xath5*, or (iii) *NeuroD*. Embryos are oriented with anterior to the left. *Xash3* was expressed in the optic vesicle of stage 21 embryos, while *Xath5* and *NeuroD* were not. Expression of *Xath5* was restricted to the prospective olfactory placodes, and *NeuroD* was expressed in the cranial ganglia.

(B) Embryos were injected with RNA at the two-cell stage then cultured until stage 13 (neural plate stage). The embryos were then stained with X-Gal to detect β-galactosidase expression (aqua) followed by whole-mount in situ hybridization (dark purple) using the DIG-labeled RNA probes described below. All embryos are shown in a dorsal view, with anterior at the top and the injected side on the right. Embryos were injected with *Xash3* (i–iii), *NeuroD* (iv), or *Xath5* (v) RNA in combination with RNA for β-galactosidase at the two-cell stage then probed for expression of *NeuroD* (i), *Xath5* (ii), *X-Ngnr-1a* (iii), or *Xash3* (iv and v). (i) *Xash3* overexpression induced ectopic *NeuroD* expression throughout the injected side but did not cause ectopic *Xath5* expression (ii) or *X-Ngnr-1a* expression (iii). Overexpression of *NeuroD* (iv) or *Xath5* (v) did not cause ectopic expression of *Xash3*, although expression of *Xash3* was often suppressed on the injected side, likely due to premature differentiation of neurons overexpressing these genes (Lee et al., 1995).

(C) Embryos were injected with RNA for β-galactosidase in combination with (i) *NeuroD* RNA, (ii) *NeuroD* plus *Xash3* RNA, or (iii) *Xash3* RNA. The embryos were probed for expression of either *N-tubulin* (i–iii) or *Xath5* (iv and v). (i) *NeuroD* overexpression induced the expression of *N-tubulin* throughout the injected side, indicating the formation of ectopic neuroepithelial cells. (ii) When *Xash3* is coinjected with *NeuroD*, expression of *N-tubulin* is suppressed on the injected side, demonstrating that *Xash3* is able to suppress the ability of *NeuroD* to promote ectopic neurogenesis. (iii) *Xash3* alone caused a reduction in the normal pattern of *N-tubulin* expression on the injected side. (iv) *NeuroD* overexpression caused activation of *Xath5* expression in the anterior neural ridge. (v) Coinjection of *Xash3* and *NeuroD* suppressed the activation of *Xath5* expression.

6Cii). Lower doses of *Xash3* had a weaker effect (data not shown). *Xash3* has been shown to activate expression of the ligand *X-Delta-1*; however, since *NeuroD* has been shown to be relatively insensitive to inhibition by Notch/Delta signaling, there may be other inhibitory factors that are activated by *Xash3* that limit the activity of *NeuroD* (Chitnis and Kintner, 1996). This inhibition could be achieved indirectly by limiting the expression of cofactors necessary for neurogenesis, such as *X-MyT1* (Bellefroid et al., 1996). It is also possible that *Xash3* limits the effects of *NeuroD* by forming nonfunctional heterodimers with *NeuroD*. *NeuroD* would then be relieved from inhibition by termination of *Xash3* expression. Recently, protein–protein interactions between Mash-1 (an achaete–scute homolog) and Math-4A (an atonal homolog) have been described, and these dimers were not able to bind to canonical E box sequences, although the functional significance of this is not clear (Gradwohl et al., 1996).

Ectopic *NeuroD* expression has previously been reported following overexpression of *X-Ngnr-1a* (Ma et al., 1996). This gene has been proposed to function upstream of *NeuroD* during primary neurogenesis in the neural plate (Ma et al., 1996). We found that overexpression of *Xash3* did not activate expression of *X-Ngnr-1a* on the injected side (Figure 6Biii, 0/13 embryos examined). This suggests that *Xash3* promotes the expression of *NeuroD* independently of *X-Ngnr-1a* activation. There may, therefore, be distinct pathways of bHLH gene activation that have different relevance in different tissues.

Overexpression of either *NeuroD* or *Xath5* did not cause ectopic expression of *Xash3* (0/52 and 0/20 embryos examined, respectively), suggesting that *Xash3* acts upstream of both genes (Figures 6Biv and 6Bv).

Overexpression of *NeuroD* or *Xath5* often caused suppression of *Xash3* expression on the injected side (Figures 6Biv and 6Bv), likely due to premature differentiation of neuroepithelial cells expressing these genes, as has been demonstrated for NeuroD (Lee et al., 1995).

Discussion

Identification of *Xath5*, a Xenopus Homolog of the Drosophila *atonal* Gene

In this study, we report the identification of a novel bHLH gene, *Xath5*, that is related to the Drosophila proneural gene *atonal* (Jarman et al., 1993). *Xath5* joins a growing family of *atonal*-related genes that share considerable sequence identity throughout the bHLH domain. Of these, *Xath5* is one of the most closely related to the Drosophila *atonal* gene, showing 100% amino acid identity in the basic domain, the region that contacts DNA and that is involved in target recognition. Although from different species, both Drosophila *atonal* and *Xath5* are expressed during retinal and olfactory development, suggesting possible conservation of downstream target genes (Jarman et al., 1994; Reddy et al., 1997). In addition, the conservation of both sequence and expression pattern between *Xath5* and Drosophila *atonal* provides further strength to the argument that the genetic hierarchy governing eye development is highly conserved (Halder et al., 1995a, 1995b).

We found that *Xath5* could promote ectopic N-tubulin and N–CAM expression throughout the ectoderm when overexpressed, an activity that has been observed for a number of other *atonal*-related genes, including *NeuroD*, *Xath3*, *NeuroD2*, *neurogenin*, and *X-Ngnr-1a* (Lee et al., 1995; Ma et al., 1996; McCormick et al., 1996; Takebayashi et al., 1997). This ability to promote neurogenesis within the ventral and lateral ectoderm may be a feature characteristic of *atonal*-related genes, since the effects on neurogenesis of the *achaete–scute*-related genes *Xash1* and *Xash3* are limited to the neural plate and immediately adjacent regions (Ferreiro et al., 1994; Turner and Weintraub, 1994). It has been suggested that the more restricted neurogenic activity of the *achaete–scute*-related gene *Xash3*, as compared to the *atonal*-related gene *NeuroD*, may represent more sensitivity to inhibitory factors within the ectoderm (Lee et al., 1995). Indeed, within the neural plate, *Xash3* is more sensitive than *NeuroD* to the inhibitory effects of the neurogenic genes *X-Delta-1* and *X-Notch-1* (Chitnis and Kintner, 1996). Recently, *X-Ngnr-1a*, but not *Xash3*, was shown to activate expression of *X-MyT1*, a gene critical for neuronal differentiation in the developing neural plate (Bellefroid et al., 1996), providing a compelling explanation for their differential ability to promote ectopic neurogenesis.

bHLH Genes Are Expressed at Two Distinct Stages of Retinal Neurogenesis and Have Different Functions

In the CMZ of the retina, the successive stages of retinal neuron development are represented, making it possible to resolve the temporal sequence of bHLH gene expression during retinal neurogenesis. We provide evidence that, in the developing retina, *Xash3* does play a distinct role from *NeuroD* and *Xath5*. *Xash3* was expressed in early retinoblasts, while *Xath5* and *NeuroD* were expressed later in differentiating retinoblasts. Therefore, the expression of these two sets of genes marked at least two distinct stages of retinal neuron development. This is analagous to the regulation of peripheral sense organ formation in Drosophila by genes of the *achaete–scute* complex. *Achaete*, *scute*, and *lethal-of-scute* are expressed early and are involved in the selection of neuronal precursors from the surrounding ectoderm, while *asense* is expressed later, within the neural precursors, and appears to be involved in their further development (Alonso and Cabrera, 1988; González et al., 1989; Brand et al., 1993). The *atonal*-related genes *Xath5* and *NeuroD* may therefore be functionally analogous to *asense*: both are expressed in proliferating cells but continue expression in postmitotic cell, and overexpression of *asense* is able to recapitulate the entire program of sense organ formation, even though it is expressed at a later step in this process, similar to the activation of neuronal development in nonneuronal cells caused by expression of *NeuroD* and *Xath5* (Brand et al., 1993; Lee et al., 1995).

Both *NeuroD* and *Xath5* were expressed in populations of differentiating cells within the CMZ, suggesting that these genes function at a similar step in the differentiation of retinal neurons. However, *Xath5* and *NeuroD* do not appear to have identical functions during retinal development, since lipofection showed that *Xath5* was able to promote retinal ganglion cell differentiation, while *NeuroD* was not. Instead, we found that lipofection of *NeuroD* biased the cells toward an amacrine or bipolar cell fate at the expense of photoreceptors and Mueller glial cells. These findings argue that *NeuroD* is not simply causing early differentiation of retinal progenitor cells, since the primary effect seems to be on intermediate or later-born cell types.

Birthdating studies in the retina have shown that cell generation occurs in two phases (Harman and Beazley, 1989; LaVail et al., 1991). First, ganglion cells, cone photoreceptors, and horizontal cells are born, then amacrine cells, Mueller glial cells, bipolar cells, and rod photoreceptors are generated. It is possible that *NeuroD* only affects this later phase of cell genesis, biasing this subset of cells toward the earlier cell types, amacrine and bipolar cells, at the expense of rod photoreceptors. Mueller glial cell differentiation is likely suppressed, since *NeuroD* tends to promote neuronal differentiation (Lee et al., 1995). There is molecular evidence for a separation in the genesis of early and late-born cell types in the retina. In rat, Mash-1 expression is restricted to the late phase of retinal neurogenesis and has been proposed to function in the development of the later-born cell types (Jasoni and Reh, 1996).

There are at least two ways that the effects of *NeuroD* might be restricted to this later phase of cell differentiation. We have shown that *Xash3* can activate expression of *NeuroD* but limit its downstream effects, possibly ensuring that *NeuroD* indeed functions only in this later wave of neurogenesis. When *NeuroD* is overexpressed in retinal progenitor cells by lipofection, it may only function once *Xash3* is degraded. Alternatively, environmental cues in the early retina could initiate inhibitory signals

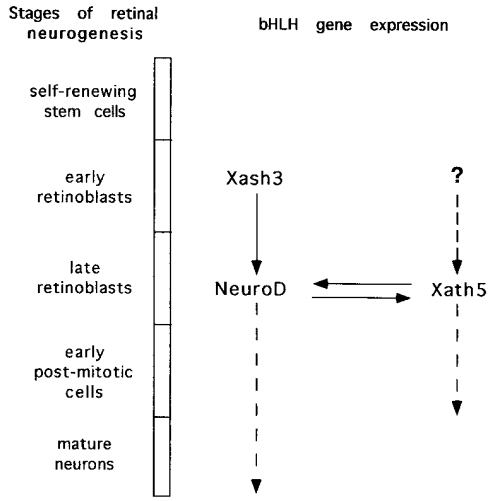

Figure 7. A Model Describing the Relationship among the bHLH Proteins Xash3, NeuroD, and Xath5 during Retinal Neurogenesis

Sequential stages of retinal neurogenesis are represented on the left. *Xash3* is expressed in proliferating retinoblasts but not stem cells, while *NeuroD* and *Xath5* are expressed in retinoblasts and early differentiating neurons. *NeuroD* continues to be expressed in subsets of mature retinal neurons. We propose that during retinal neurogenesis, *Xash3* induces expression of *NeuroD* but suppresses its downstream effects until *Xash3* expression is terminated. Unknown parallel mechanisms may activate expression of *Xath5*; however, the coexpression of *NeuroD* and *Xath5* could be maintained by their ability to cross-activate each other's expression. *NeuroD* and *Xath5* may then function either together or in parallel to regulate retinal cell differentiation.

that limit the function of *NeuroD* until later. Further studies will be needed to establish whether either of these models is correct.

In the central retina, *NeuroD* expression persists in differentiated cells of the outer nuclear layer, whereas *Xath5* is no longer expressed. *NeuroD* may therefore be involved not only in the development of retinal neurons but also in the maintenance of their differentiated phenotype, while *Xath5* may function only to regulate differentiation. Not all mature retinal neurons continue to express *NeuroD*, raising the possibility that other bHLH genes are involved in maintaining the differentiated state of these other cells.

The Expression of *Xash3*, *NeuroD*, and *Xath5* Can Be Coupled

The expression of *Xash3*, *NeuroD*, and *Xath5* suggests that these genes regulate at least two distinct stages of retinal neuron differentiation. Our results show that they are capable of forming a regulatory cascade that could couple the sequential steps of retinal neurogenesis (summarized in Figure 7). We found that overexpression of *Xash3* could activate expression of *NeuroD*, possibly explaining the sequential activation of these genes during retinal neurogenesis. Although *Xash3* could activate expression of *NeuroD*, it was also able to block dominantly the downstream effects of *NeuroD* activation. This might reflect similar events during normal development and suggests that *Xash3* could limit the ability of retinal progenitor cells to respond to *NeuroD* expression until *Xash3* expression is terminated. *Xash3* may therefore play a role in regulating the timing of the transition from retinal precursor cell to differentiated retinal neuron, thus serving to confer competence on the progenitor cells without actually promoting their differentiation. During the development of autonomic progenitor cells in fetal rat, Lo et al. (1997) have shown that *Mash-1* expression maintains the progenitor cells in a state competent to undergo neurogenesis in response to BMP2 signals but is not itself sufficient to promote neuronal differentiation (Lo et al., 1997).

NeuroD and *Xath5* were found to be coexpressed during retinal cell differentiation, and this coexpression might be ensured by their ability to cross-activate each other's expression. This does not exclude the possibility that other factors regulate the pattern and sequence of bHLH gene expression within the developing retina. Given that both *NeuroD* and *Xath5* commence expression at the same stage of retinal cell differentiation, it seems possible that the coexpression of *NeuroD* and *Xath5* is first governed by common activation of the same *cis*-regulatory elements in both genes by an upstream regulatory factor. During Drosophila sense organ formation, this is how the coexpression of *achaete* and *scute* in proneural clusters is achieved (Gómez-Skarmeta et al., 1995). However, the ability of *NeuroD* and *Xath5* to cross-activate each other's expression may be a means of reinforcing their coexpression during the differentiation of retinal neurons.

NeuroD has been shown to participate in a different regulatory cascade during primary neurogenesis in the neural plate of Xenopus embryos (Ma et al., 1996). The neural plate region differs from the developing retina in that *Xash3* and *NeuroD* are not expressed sequentially within the same population of cells (Anderson, 1995). Rather, *NeuroD* expression is preceded by *X-Ngnr-1a*, and overexpression of *X-Ngnr-1a* causes ectopic expression of *NeuroD* (Ma et al., 1996). Therefore, in the developing neural plate, *X-Ngnr-1a* and *NeuroD* likely regulate successive stages in the development of primary neurons (Ma et al., 1996). We found that overexpression of *Xash3* caused ectopic *NeuroD* expression but did not alter *X-Ngnr-1a* expression. Therefore, there may be at least two independent ways to regulate the expression of *NeuroD*, possibly depending upon the expression of specific cofactors, and different pathways of activation may be used in different parts of the developing nervous system. This means that in the developing neural plate, *Xash3* may activate target genes other than *NeuroD*, since these genes are expressed in separate lineages. Whether this is important for determining regional identity or neuronal cell type has yet to be established.

Based upon the findings of this study, we suggest that a network of bHLH genes, including *Xash3*, *NeuroD*, and *Xath5*, functions to regulate the sequential steps of retinal neurogenesis (Figure 7). We suggest that *Xash3* functions in early retinoblasts and may then activate expression of *NeuroD*. *Xath5* was coexpressed with *NeuroD*, and this coexpression may be maintained by their ability to cross-activate each other's expression. *NeuroD* and *Xath5* may then function either in parallel or in combination to regulate the differentiation of retinoblasts into postmitotic neurons. Since the evidence

for these regulatory interactions is based upon gain-of-function experiments, it will be necessary to interfere with the activity of *Xash3*, *NeuroD*, and *Xath5* during normal development to provide evidence that these genes play necessary roles in the regulation of retinal neurogenesis. In addition, since other bHLH genes are known to be expressed in the developing retina (M. P. and W. A. H., submitted), additional players are likely to add to the complexity of these regulatory interactions.

Experimental Procedures

Molecular Cloning of *Xath5* cDNA

Fully degenerate PCR primers were designed based upon two amino acid sequences conserved between the bHLH domains of Math1 and Drosophila atonal: RRLAANA (GI[CA]GI[CT]TIGCIGCIAA[CT]GC) and ETLQMAQ (TGIGCCAT[CT]TGIA[AG]IGT[CT]TC) (Jarman et al., 1993; Akazawa et al., 1995). Twenty-five picomoles of each primer were used in a 25 μl reaction with 1 μl of a stage 28–30 Xenopus head cDNA library in λZAPII (2.5×10^{10} pfu/ml) included as template. The library was a generous gift from R. Harland (Hemmati-Brivanlou et al., 1991). Forty cycles of PCR were performed with an annealing temperature of 60°C. PCR products were subcloned using a TA cloning kit (Invitrogen) then sequenced using standard methods (Sequenase/USB). Two highly similar PCR products were identified. One of these was used to probe the same stage 28–30 Xenopus head cDNA library. Two sets of cDNAs encoding *atonal*-related proteins were isolated, and the longest were sequenced in their entirety on both strands by automated sequencing (University of Utah Core Facility). One 2.14 kb cDNA encoded *Xath5a*, and the other 2.29 kb cDNA encoded *Xath5b*.

The *Xath5a* and *Xath5b* were subcloned into the EcoRI and XbaI sites of the expression vector CS2+ (Turner and Weintraub, 1994) by PCR amplifying the coding regions using Pfu polymerase. The 5′ primers used were either GCGCGAATTCGGGATAGAATATCTCT GCTG (EcoRI site underlined; 22 bp upstream of the ATG of *Xath5a*) or GCGCGAATTCGCCATAGAATCACTGTGTTG (EcoRI site underlined; 22 bp upstream of the ATG of *Xath5b*), and the 3′primer used was GCGCTCTAGA**CTA**GTGAGAAAAAGTCTGGGGC (XbaI site underlined, stop codon in bold). All subclones that were generated by PCR were fully sequenced by automated sequencing (University of Utah Core Facility) and were tested for the ability to produce protein of the predicted size by in vitro translation (TNT Coupled Reticulocyte Lysate system, Promega).

In Situ Hybridization and Immunohistochemistry

Digoxigenin (DIG)-labeled antisense RNA probes were generated for *Xath5a* and *Xath5b*, *NeuroD* (Lee et al., 1995), *Xash3a* (Turner and Weintraub, 1994), *X-Ngnr-1a* (Ma et al., 1996), and *N-tubulin* (Richter et al., 1988) as described (Harland, 1991). A fluorescein-labeled *Xath5a* antisense RNA probe was also generated for use in double in situ hybridizations. Whole-mount in situ hybridization was performed on albino Xenopus embryos using the procedure described (Harland, 1991), except that BM purple (Boehringer Mannheim) was used as the substrate for the alkaline phosphatase, since the background was lower than that obtained using NBT/BCIP. After in situ hybridization, some embryos were cleared using a 2:1 mixture of benzyl benzoate/benzyl alcohol (Dent et al., 1989).

In situ hybridizations on 10 μm paraffin sections of Xenopus embryos were done essentially as described (Dorsky et al., 1995), except that PBT (PBS, 0.2% bovine serum albumin, 0.2% Triton X-100) was replaced with MAB (0.1 M maleic acid, 0.15 M NaCl [pH 7.5]) and the PBT/20% normal goat serum was replaced with MAB/2% BMBR (Boehringer Manheim blocking reagent). Double in situ hybridizations using DIG-labeled *NeuroD* probe and fluorescein-labeled *Xath5a* probe were done essentially as described (Dorsky et al., 1997). However, instead of inactivating the remaining alkaline phosphatase after the first signal was obtained, the anti-fluorescein antibody bound to the fluorescein-labeled probe was removed in glycine HCl (pH 2.2) for 10 min, and sections were then washed five times in PBS. The DIG-labeled *NeuroD* RNA probe was detected using NBT/BCIP substrate (Boehringer), and the fluorescein-labeled *Xath5* RNA probe was detected using fast red substrate (Boehringer). To visualize nuclei, the sections were then stained with Hoechst dye (0.5 μg/ml) for 10 min, rinsed three times in PBS, and then mounted under coverslips.

Immunohistochemistry was performed using an anti-N–CAM antibody (gift from Urs Rutishauser) diluted 1:100 using methods described previously (Turner and Weintraub, 1994). The primary antibody was detected with a horseradish peroxidase-conjugated sheep anti-rabbit antibody (Jackson Laboratories) diluted 1:5000, followed by reaction with diaminobenzidine (DAB).

Microinjection of RNA

Capped RNA was synthesized in vitro by SP6 transcription from pCS2-*Xath5*, pCS2MT-*XNeuroD*, pCS2MT-*Xash3*, or pCS2-*nuclear* β*galactosidase* (*n*β*gal*) template DNA using a Message Machine kit from Ambion. For two-cell stage injections, RNA was injected in a volume of 5 nl into one of the two blastomeres in the following amounts: *Xath5* (1 ng), *NeuroD* (0.6 ng), *Xash3* (0.7 ng), and *n*β*gal* (0.1 ng). Since the first cleavage plane often defines the plane of bilateral symmetry in the embryo, the cells in one half of the embryo express the injected RNA, and the other half of the embryo serves as an uninjected control. The embryos were allowed to develop and were then staged according to Nieuwkoop and Faber (1994). The embryos were fixed in MEMFA (Harland, 1991) for 2 hr at room temperature then stored in methanol. X-Gal staining was performed on embryos injected with β-galactosidase RNA as previously described (Turner and Weintraub, 1994). For 16-cell stage injections, *Xath5* RNA (100–150 pg in a volume of 1 nl) was injected into blastomere D.1.1. GFP RNA (50 pg) was coinjected to follow the cells derived from the injected blastomere. Embryos were fixed at stage 41, sectioned on a cryostat at a thickness of 16 μm, then the sections were stained with Hoechst (30 μM in PBS) to visualize the nuclei. Cells misexpressing GFP were counted and the cell type identified based upon their laminar position and morphology, as previously described (Dorsky et al., 1995, 1997).

In Vivo Lipofection

DNA was transfected into the anterior neural folds of stage 18 embryos as previously described (Holt et al., 1990; Dorsky et al., 1995). GFP DNA was cotransfected to mark the transfected cells. Embryos were fixed at stage 41, sectioned on a cryostat at a thickness of 16 μm, then the sections were stained with Hoechst (30 μM in PBS) to visualize the nuclei. Cells misexpressing GFP were counted and the cell type identified based upon their laminar position and morphology, as previously described (Dorsky et al., 1995, 1997). Expression of *myc* tagged Xath5 protein was confirmed in some sections by immunostaining using the 9e10 mononclonal antibody.

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